Original Article

Global micro-RNA expression induced by tetramethylpyrazine treatment for spinal cord injuries

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Abstract: Spinal cord injuries (SCI) cause serious vascular dysfunction. Tetramethylpyrazine (TMP) treatment has been shown to promote angiogenesis and improve locomotor and neurological function after SCI. However, mechanisms underlying the effects of TMP on SCI remain unclear. The aim of the present study was to uncover the influence of TMP treatment on microRNA (miRNA) expression. Next-generation sequencing was used to analyze global gene expression changes in miRNAs induced by TMP. Real-time PCR (RT-PCR) assay was used to verify data. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were performed using the Database for Annotation, Visualization, and Integrated Discovery tool, aiming to predict the functions of target genes of differentially-expressed miRNAs. A rat model of SCI was constructed using a modified version of Allen’s impact method. Adult Sprague-Dawley rats were randomly allocated to the following four groups, with 6 rats in each group: (A) Control group; (B) Sham operation group; (C) SCI group; and (D) TMP-treated SCI group. In Group D, the rats were injected intraperitoneally with TMP. Total RNA was isolated from spinal cord tissues from Group C and Group D at 5 days after SCI, allowing for sequencing of miRNA expression profiles. Compared with Group C, miRNAs in Group D were enriched in complement and coagulation cascades, systemic lupus erythematosus, and prion disease pathways. After TMP treatment, some miRNAs, including rno-miR-10-5b, X_119707, 13_8820, and rno-miR-375-3p, were upregulated, while 7_5613 and rno-miR-6324 were downregulated. This was confirmed by RT-PCR results. In addition, many novel miRNAs were predicted by the miRDeep2 algorithm. Collectively, present results suggest that TMP treatment after SCI influenced the expression levels of some miRNAs. Additional studies concerning these miRNAs are necessary, aiming to improve the understanding of mechanisms underlying the effects of TMP on SCI.

Keywords: MicroRNA, tetramethylpyrazine, spinal cord injury

Introduction

Spinal cord injuries (SCI) result in major vascular trauma [1-4]. Symptoms of SCI vary from pain to paralysis and incontinence. SCI causes secondary damage and pathological changes, including edema, axonal degeneration, hemorrhaging, and neuronal necrosis, as well as demyelination followed by cyst formation and infarction [5, 6]. These changes affect cell survival and neurological integrity via complex and evolving molecular cascades. However, these interrelationships are not fully understood. SCI-induced pathophysiological alterations may last for several years due to strong inhibition of neuronal precursors and neurogenesis, leading to paralysis [7]. Many studies have focused on the development of effective treatments for functional deficits in SCI patients [8]. Several kinds of therapeutic approaches have been carried out, aiming to improve the microenvironment and stimulate endogenous repair, and thus ameliorate secondary injuries. However, a lack of scientific understanding of the mechanisms of SCI has caused treatment of this disease to remain a major challenge for clinicians.

In recent years, many studies have investigated the effects of microRNAs (miRNA) on SCI [9]. Some studies have reported that miRNAs appear to mediate neural plasticity, suggesting that they are possibly involved in neurodegeneration and regulation of gene expression after...
SCI [10, 11]. MiRNAs, a class of noncoding RNAs, have around 22 nucleotides on average. They play important roles in the regulation of many cellular processes, including cell proliferation and apoptosis, via post-regulation of gene expression [12]. They also control mRNA expression by directly binding to target mRNAs, resulting in either degradation of the target mRNA or inhibition of translation [13]. Previous studies have shown that expression levels of miRNAs are altered in association with secondary injuries in SCI, suggesting that miRNAs may play an important role in SCI [14].

A previous study demonstrated tetramethylpyrazine (TMP), a phosphodiesterase inhibitor, is effective in accelerating functional recovery of acute SCI in a rat model [15]. In China, TMP has been widely used for treatment of cardiovascular disease and SCI [16-18]. However, cellular mechanisms underlying the activity of TMP remain unclear.

The present study, aiming to explore mechanisms underlying the effects of TMP on SCI, established a rat SCI model. This study used next-generation sequencing, examining the effects of TMP on miRNA expression profiles.

Material and methods

Animals and experimental design

Adult Sprague-Dawley rats, weighing 180 to 200 g, were maintained in a temperature-controlled and light-controlled (12-hour light/12-hour dark cycle) room with food and water. Experimental protocols were performed in accordance with National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. The current study was approved by the Animal Care and Research Committee of Shanghai University of Traditional Chinese Medicine University. The rats were randomly allocated to the following four groups, with six rats in each group: (A) Control group; (B) Sham operation group; (C) SCI group; and (D) TMP-treated SCI group. The Basso, Beattie, and Basso-Beattie-Bresnahan (BBB) locomotor scale [19, 20] was used to assess functional consequences at 24 hours, 4 days, and 5 days after the induction of SCI, as well as to reveal the effects of TMP on recovery. Zero referred to no observable hind limb movement and 21 referred to normal rat locomotion. Due to economic reasons, tissue samples were collected only from Group C and Group D at 5 days after SCI for sequencing of miRNA expression profiles.

SCI rat model

The rat model of SCI was created based on the Allen weight-drop method [21]. The rats were anesthetized with chloral hydrate (450 mg/kg) by intraperitoneal injections. A laminectomy was carried out at the thoracic vertebrae 10 (T10), exposing the spinal cord beneath without disruption of the dura. Next, the spinous processes of T8 and T11 were fixed. This was followed by exposed T10 being submitted to weight-drop contusion injuries, as previously described [22]. Subsequently, the muscles and skin were closed in layers. In the control group, no surgeries were performed. In the sham operation group, the animals underwent a T10 laminectomy without weight-drop injury. Rats in the TMP-treated SCI group were treated with intraperitoneal injections of TMP (200 mg/kg daily) after surgery.

RNA isolation

The rats were euthanized by pentobarbital-phenytoin overdoses (1 mL IP per rat) 5 days after SCI. The spinal cord was exposed and 10 mm of the spinal cord was harvested. Total RNA was isolated from the collected tissues using the RNeasy Mini Kit (Qiagen, West Sussex, UK), according to manufacturer protocol. Briefly, 500 µL of lysis buffer was added to each sample. After homogenizing with a QIASHredder (Qiagen, West Sussex, UK), RNA was extracted. The quality of RNA in all samples was confirmed using an Agilent 2100 BioAnalyzer (Agilent Technologies, Palo Alto, CA).

MicroRNA sequencing and data analysis

RNA sequencing was performed on a polyadenylated fraction of RNA isolated from the spinal cord, with 3 samples in each group. Only samples with RNA integrity numbers (RIN) > 8 were used for library construction. Moreover, 150-300 ng of total RNAs obtained from the aforementioned experiment were used for the sequencing library. After the RNA samples were selected by polyA, paired-end sequencing libraries were built using a TruSeq RNA Sample Prep Kit (Illumina, San Diego, USA), as described in the TruSeq RNA Sample Preparation
V2 Guide. Next, the samples were sequenced with an Illumina HiSeq sequencer. Differentially-expressed miRNAs (P < 0.05) were identified via volcano plot filtering.

**Gene ontology (GO) enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis**

Putative targets of the differentially-expressed miRNAs were predicted using TargetScan, miRanda, and PicTar online software. GO and KEGG pathway analyses of predicted target genes of differentially-expressed miRNAs were performed using the Database for Annotation, Visualization, and Integrated Discovery tool (http://david.abcc.ncifcrf.gov/) [23]. Only differentially-expressed miRNAs with P < 0.05 and false discovery rates (FDR) < 0.05 were included in this study.

**RNA preparation and real time quantitative PCR (RT-PCR)**

After total RNA was extracted using TRIzol Reagent (Thermo Fisher Scientific, MA, USA), the TaqMan MicroRNA Reverse Transcription Kit (Takara, Dalian, China) was used for miRNA detection. RT-PCR was performed, assessing expression levels of miRNAs using TaqMan miRNA qPCR kits (HaiGene, Heilongjiang Province, China) with specific primers, using U6 snRNA as control on the ABI 7500 Sequence Detection System (Thermo Fisher Scientific). Expression levels of miRNAs were calculated using the 2^ΔΔCt method.

**Statistical analysis**

All experiments were repeated three times. One-way analysis of variance (one-way ANOVA) and t-tests were applied, determining the significance of the data. Data are presented as mean ± standard deviation (SD). Defining significant correlation levels, Pearson's correlation coefficient was employed. A correlation coefficient of > 0.5 or < -0.5 was accepted as statistically significant. After filtering, miRNAs that met this criterion were subjected to GO enrichment analysis.

**Results**

**TMA treatment contributes to functional recovery after SCI**

Effects of TMA on locomotor function were assessed using the BBB test, aiming to explore whether the drug promoted functional recovery after SCI. As shown in Table 1, compared with group B, 24 hours after SCI, all injured rats in groups C and D were paralyzed in their hind limbs with reduced BBB scores (P < 0.05). This suggested that the SCI model was generated successfully. In addition, BBB scores were significantly increased in TMA treated rats at 4 days and 5 days after SCI, compared to rats in group C (P < 0.05), suggesting that TMA treatment improved the functional recovery of rats after SCI.

**Effects of TMP treatment on miRNA expression in the SCI rat model**

Investigating the effects of TMP treatment on SCI and the relationship of TMP with miRNA expression profiles, next generation sequencing was used to determine miRNA expression levels in rats. After filtering and mapping to the reference genome, 109 miRNAs were up-regulated, including rno-miR-10-5b, X_119707, 13_8820, and rno-miR-375-3p. Moreover, 109 were downregulated, including 7_5613 and rno-miR-6324 (Log2 [fold changes < 1 or > -1; Figure 1), in TMP-treated SCI rats, compared with the non-TMP treatment group. Results suggest that TMP treatment affected the expression profiles of miRNAs.

**Function analysis of differentially-expressed miRNAs**

Aiming to identify the biological pathways in which differentially-expressed miRNAs were involved, miRNAs were characterized based on reference pathways in KEGG. KEGG enrichment analysis showed that differentially-expressed miRNAs were significantly associated with three pathways (complement and coagulation...
TMP treatment on miRNA expression after SCI

Figure 1. Overview of differences in global miRNA expression before and after TMP treatment. A. Scatter plot of mean expression quantity between SCI and TMP-treated SCI groups; B. Volcano plot showing differentially-expressed miRNAs between SCI and TMP-treated SCI groups.
cascade, prion disease, and systemic lupus erythematosus) ($P < 0.05$), as shown in Figure 2. This finding suggests that TMP treatment affected expression of miRNAs involved in secondary injuries in a rat model of SCI, indicating that the effects of TMP on functional recovery after SCI might be through attenuation of secondary injuries. To further understand the function of differentially-expressed miRNAs, GO annotation was performed. Differentially-expressed miRNAs were assigned to at least one term in several GO categories, including biological process, cellular component, and molecular function. These miRNAs were then classified into several subcategories in terms of function. The top 10 items from each category were...
TMP treatment on miRNA expression after SCI

selected (Figure 3). In the biological process category, the protein activation cascade was the largest group. In the cellular component category, membrane attack complex represented the largest function group. In the molecular function category, activation transcription factor binding was the dominant function group. Present findings suggest that TMP treatment mainly affected miRNAs involved in the regulation of transcription and protein activation. The latter could possibly lead to a change in expression levels of specific proteins involved in secondary injuries in SCI.

Identification of differentially-expressed miRNA

Alignment analysis performed against the global data set. It revealed that rno-miR-10-5b, X_119707, 13_8820, rno-miR-375-3p, and rno-miR-496-3p were upregulated in TMP-treated rats. In agreement with previous studies [24-26], levels of several miRNAs (miR-21a, miR-130b, miR-19a, miR-34a, miR-142a, miR-199a, miR-184, and miR-147) were significantly downregulated in the TMP-treated group after SCI (Figure 4A). Subsequently, RT-PCR confirmed the expression patterns of these differentially-expressed miRNAs in TMP-treated SCI group and SCI group. Results showed that expression levels of rno-miR-10-5b, X_119707, 13_8820, rno-miR-375-3p, and rno-miR-496-3p were upregulated, while expression levels of miR-21a, miR-130b, miR-19a, miR-34a, miR-142a, miR-199a, miR-184, and miR-147 were reduced in the TMP-treated SCI group, compared with the SCI group (Figure 4B).

Recent advances in high-throughput sequencing have enabled the detection of miRNAs with unprecedented sensitivity [27-29]. However, the computational task of accurately identifying miRNAs from the background of sequenced RNAs remains challenging. To aid this task, the miRDeep2 algorithm has been developed, according to a previous study [30]. This algorithm identifies canonical and noncanonical miRNAs, such as those derived from transposable elements. It selects candidates with high confidence detected in multiple independent samples (Figure 5). In the present study, this algorithm identified many novel differentially-expressed miRNAs caused by TMP (Table 2). Further studies are necessary to shed light on the function of these novel miRNAs in SCI.

Identification of potential targets of differentially-expressed miRNAs

In addition to expression pattern analysis, a search of miRNA databases was conducted, aiming to identify potential targets of upregulated miRNAs (X_119707, rno-miR-375-3p, 13_8820, rno-miR10b-5p, and mi-miR-496-3p). The search revealed numerous genes, including Gad1, Alx4, Cblb1, Cd82, and Pcfg3, targeted by upregulated miRNAs (Figure 6).

Discussion

SCI has far-reaching effects on patient lives. Although TMP has been widely used for treatment of SCI, the mechanisms underlying its activity remain unclear. The present study investigated the effects of TMP on SCI, focusing mainly on changes in miRNA expression levels, assessed by next-generation sequencing.
Results demonstrated that several miRNAs were significantly upregulated or downregulated after TMP treatment in a rat model of SCI. Some of the identified miRNAs had been detected in previous studies, indicating that present data and present experiments were reliable and consistent. In addition, some novel miRNAs were identified. Further studies of these miRNAs may aid the understanding of SCI.

Thousands of miRNAs have been identified since the first report of miRNAs 10 years ago [9]. Increasing evidence has shown that miRNAs play an important role in a variety of biological processes, such as cell differentiation, cell proliferation, cell growth, and metabolism, largely by inhibiting mRNA translation [31]. A recent study demonstrated that aberrant expression of miRNAs was induced after SCI in a rat model [32]. In addition, deregulation of...
Table 2. Novel miRNAs in this study

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Figure 6. Prediction of miRNA target genes.

TMP treatment on miRNA expression after SCI
miRNAs expression plays crucial roles in the pathogenesis of secondary injuries after SCI. Furthermore, studies have confirmed that downregulation of some miRNAs induces abnormal development of the spinal cord, indicating the vital role of miRNAs in cases of SCI [33]. In the present study, aiming to investigate the mechanisms of TMP, next-generation sequencing was used to determine miRNA expression levels in a rat model of SCI, with and without TMP treatment. Results revealed 109 upregulated miRNAs and 109 downregulated miRNAs after TMP treatment. These differentially-expressed miRNAs were significantly enriched in three KEGG pathways (complement and coagulation cascade, prion disease, and systemic lupus erythematosus), which may affect expression levels of genes involved in SCI-induced secondary injuries. Present findings suggest that the effects of TMP on functional recovery after SCI might occur through attenuation of secondary injuries.

People with SCI are at risk of developing type II diabetes, either due to resistance to the effects of insulin or an inability to maintain normal glucose levels because of insufficient insulin [34]. In the present study, mir130b was downregulated after TMP treatment. According to miRGate predictions, Insulin 2 (Ins2) is the predicted target gene of mir130b [35]. Ins2 encodes pre-proinsulin, which forms insulin, the hormone involved in glucose and lipid metabolism [36]. It was proposed that, after TMP treatment, downregulation of miR-130b may lead to upregulation of Ins2, thus triggering insulin production and the maintenance of normal levels of glucose. This decreases the risk of developing type II diabetes after SCI. A better understanding of the effects of downregulated miRNAs may aid in furthering the understanding of the mechanisms of TMP.

Previous studies have shown that miR21 is upregulated after SCI, which directly targets programmed cell death protein 4, Fas ligand (a pro-apoptotic factor), and phosphatase and tensin homolog, a strong antiapoptotic factor [37]. Studies have also shown that knockdown of miR-21 hampers the recovery of hind limb motor function, resulting in increased lesion size and decreased tissue sparing, suggesting a role for miR-21 in protection and recovery [38]. Another study found that short-term exercise resulted in a dramatic increase in miR-21 expression after SCI, thus reducing functional and structural deficits after the primary injury [39]. Downregulation of miR-21 after TMP treatment indicates that TMP may have a similar effect as miR-21 (inhibition of apoptosis). Thus, it may negatively regulate expression levels of miR-21. However, more studies are necessary, aiming to shed light on the mechanisms of TMP, including potential apoptotic effects.

The present study had several limitations, including a small sample size, lack of functional analysis and validation of differentially-expressed miRNAs, and broad impact of miRNAs on many genes.

In conclusion, the current study demonstrated that TMP treatment after SCI altered the expression levels of miRNAs. This may contribute to the protective effects of TMP. Aberrantly-expressed miRNAs were enriched in complement and coagulation cascade, prion disease, and systemic lupus erythematosus pathways, indicating the effects of TMP on SCI-induced secondary injuries. In addition, some miRNAs identified in the present study were involved in apoptosis pathways, pointing to antiapoptotic effects of TMP on SCI. Present finding suggest that the protective effects of TMP on SCI might occur by attenuating secondary injuries or exerting anti-apoptotic effects. Additional studies concerning these differentially-expressed miRNAs after TMP treatment are necessary, obtaining a better understanding of the mechanisms of TMP on SCI.

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Disclosure of conflict of interest

None.

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